Substrate specificity of the widely expressed subtilisin-like proprotein convertases (SPCs): A role for the substrate amino acid directly downstream of the endoproteolytic cleavage site

by

Larry D. Tippett

Thesis submitted to the Faculty of the

University of Michigan-Flint

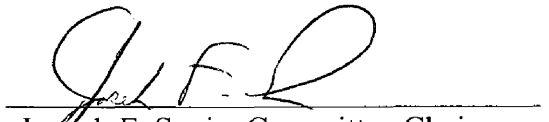
in partial fulfillment of the requirements for the degree of

Master of Science

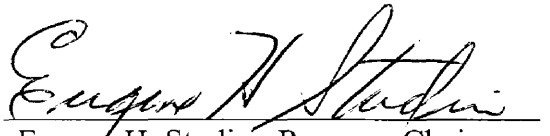
in

Biology

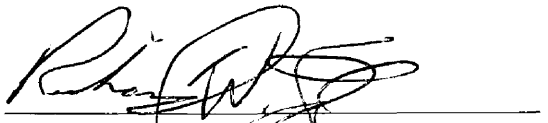
APPROVED:



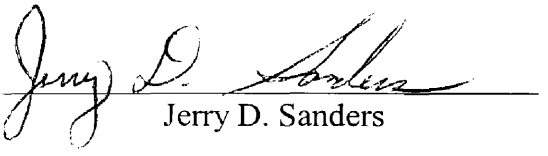
Joseph F. Sucic, Committee Chairman



Eugene H. Studier, Program Chairman



Richard W. Frazee



Jerry D. Sanders

August 2001

Flint, Michigan

Substrate specificity of the widely expressed subtilisin-like proprotein convertases (SPCs): A role for the substrate amino acid directly downstream of the endoproteolytic cleavage site

by

Larry D. Tippett

Joseph F. Sucic, Chairman

Biology

ABSTRACT

Substrate specificity was investigated among four of the eukaryotic, widely expressed subtilisin-like proprotein convertases (SPCs): furin, PACE4, PC5, and PC7. Through site-directed mutagenesis, amino acid substitutions in the P1' position of pro-von Willebrand Factor (pro-vWF), a proprotein processed by each SPC, were made by incorporating three different hydrophobic residues at this site: alanine, phenylalanine, and glycine. Results from *in vivo* and *in vitro* studies demonstrate that the presence of a P1' phenylalanine significantly impairs cleaving ability of furin, PACE4, and PC5. These results suggest the residue residing in the P1' position does not solely determine substrate specificity of these SPCs. PC7 showed approximately equivalent levels of activity versus wild type pro-vWF and all three pro-vWF mutant substrates, possibly indicating a broader substrate range for PC7.

Acknowledgements

First and foremost, I thank my wife, Lisa Brownell, for her support, understanding, and patience during this research. Without her, this thesis would not have been possible. I also thank Lisa for the plasmid sketches found in Figures 3 and 4. I thank my family as well, for their continual support, encouragement, and love. Next, I thank my major professor, Dr. Joseph Sucic, for allowing me to work in his laboratory. Over the course of research that led to this work, I have gained the highest respect for his patience, professionalism, dedication, and understanding. I have had a very rewarding experience working with him. I also wish to thank members of the "Sucic lab" team who helped make this work possible: Holly Sucic, for useful suggestions, review of this body of work, and for DNA sequencing; Gerald Cummings, for assisting with my experiments while I was teaching; Kristin Hockman, for the secreted form of furin she developed; Beth Ziemba, for cells she transfected to stably express PC7, and subsequent PC7 membrane fractions. I thank Dr. Richard Frazee, of the University of Michigan-Flint Chemistry Department, for his helpful discussions and suggestions throughout this research. Next, I thank Dr. Eugene Studier, for leading the Biology Department in the development of the Master of Science program at the University of Michigan-Flint. I also thank my committee members for their guidance and advice. Finally, I thank the Office of Research for the Research Initiatives Partnership Award, and the Biology Department at the University of Michigan-Flint, for funding this work.

Table of Contents

List of Figures

List of Tables

Introduction

In eukaryotic cells, polypeptides are produced at free cytoplasmic ribosomes or at ribosomes bound to the endoplasmic reticulum (e.r.). Proteins translated on the latter are transported into the constitutive secretory pathway, which is composed of the e.r., Golgi apparatus, trans-Golgi network (TGN), and secretory vesicles. These proteins may remain localized within the secretory system, may enter secretory vesicles and be released from the cell, or may move to the cell surface. Proteins that enter the secretory pathway typically undergo extensive post-translation modification. These post-translational modifications include glycosylation, phosphorylation, and endoproteolysis. For general overviews of secretory system protein trafficking and modifications see Lewin (2000) or Alberts et al. (1994).

Endoproteolysis, the sequence-specific hydrolysis of one or more peptide bonds in a protein, is of critical regulatory significance. Many proteins are activated as a result of endoproteolysis. The inactive precursor protein is usually referred to as a proprotein or zymogen. Many proproteins are activated by endoproteolytic removal of a short stretch of amino acids at the amino terminus called the propeptide; however, an activating endoproteolytic event may occur at any position in a protein (Alberts et al., 1994). Endoproteolysis is catalyzed by endoproteases, a group of enzymes that process proproteins into their mature, functional form. The regulatory significance of endoproteolysis and endoproteases has been known for decades; for example, blood clotting has been shown to involve a sequential cascade of endoproteolytic events (reviewed by Kalafatis et al., 1997). Many proteins that enter the secretory pathway, such

as hormones, growth factors, receptors, and extracellular matrix proteins, have been discovered to require endoproteolytic activation (reviewed by Barr, 1991; Steiner et al., 1992).

Little was known about the nature of secretory system endoproteases until the discovery and cloning of the *KEX2* gene of *Saccharomyces cerevisiae*. The product of this gene, Kex2p, also referred to as kexin, was found to share amino acid sequence similarity within its catalytic domain to bacterial serine proteases, which belong to the subtilisin family (Fuller et al., 1988, Mizuno et al., 1988). The physiological role of kexin was determined to be processing and activation of α-mating factor and killer toxin, both activated through intercellular cleavage of their proproteins at paired basic amino acid residues (Fuller et al., 1988). Discovery of *KEX2* led to identification of the first mammalian homologue, which was discovered upstream of the *fes/fps* protooncogene on human chromosome 15. The gene, termed *fur* (for c-***fes/fps*** **u**pstream **r**egion), coded for a putative protein designated furin (Fuller et al., 1989; Roebroek et al., 1986a; Roebroek et al. 1986b), which was subsequently shown to be a subtilisin-like serine endoprotease (Wise et al., 1990; Bresnahan et al., 1990; Van de Ven et al., 1990).

Following the discovery and characterization of the *fur* gene and its product, experimentation using polymerase chain reaction (PCR) methods led to the discovery of a family of six *fur*-like genes in higher eukaryotes (for reviews see Nakayama, 1997; Seidah and Chretien, 1997; Steiner, 1998). Products of these genes are: PC1/PC3 (for **p**rohormone or **p**roprotein **c**onvertase; hereafter referred to as PC1), PC2, PACE4 (for **p**aired basic **a**mino acid **c**leaving **e**nzyme 4), PC4, PC5/PC6 (hereafter referred to as PC5), and LPC/PC7/PC8/SPC7 (hereafter referred to as PC7). These enzymes are

collectively termed the SPCs (for subtilisin-like proprotein convertases). PC1, PC2, and PC4 exhibit a limited pattern of expression. PC1 and PC2 are expressed only in neuroendocrine tissue, while PC4 is expressed only in testicular spermatogenic cells (reviewed by Nakayama, 1997; Seidah and Chretien, 1997; Steiner, 1998). Furin, PACE4, PC5, and PC7 are widely expressed in diverse tissues and cell lines, and are believed to function in the constitutive secretory pathway of many different mammalian cell types (reviewed by Nakayama et al., 1997; Seidah and Chretien, 1997; Steiner 1998).

The work described herein focuses on these widely expressed SPCs. Collectively, they can endoproteolytically process and activate a number of proproteins that move through the secretory pathway. These proproteins include growth factors, hormones, receptors, plasma proteins, and proteins of the extracellular matrix (Nakayama et al., 1997; Seidah et al., 1997; Steiner 1998, and references therein). In addition, these SPC enzymes also activate proteins that are involved in the development of disease conditions, including viral envelope glycoproteins and bacterial exotoxins (reviewed by Nakayama et al., 1997; Seidah et al., 1997; Steiner 1998). Recently, these SPC enzymes have been implicated in the activation of proteins involved in tumor progression and metastasis (Bassi et al., 2000).

All SPCs share similar structural features, as shown in Fig. 1 (reviewed by Nakayama et al., 1997; Seidah et al., 1997; Steiner 1998). Located at the amino terminal end is the signal peptide (SP), which facilitates docking of ribosomes to the e.r. and directs transport of nascent polypeptides into the e.r. The propeptide (PRO) is autocatalytically removed through cleavage at an N-Arg-X-Lys-Arg-C site during

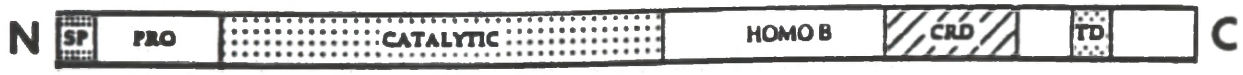


Figure 1A. **Structural components of furin (modified from Spence et al. 1995).** Regions of furin, from amino-terminus to carboxy-terminus, are as follows: signal peptide (SP), propeptide (PRO), catalytic domain (CATALYTIC), Homo-B domain (HOMO B), cysteine-rich domain (CRD), and transmembrane domain (TD).

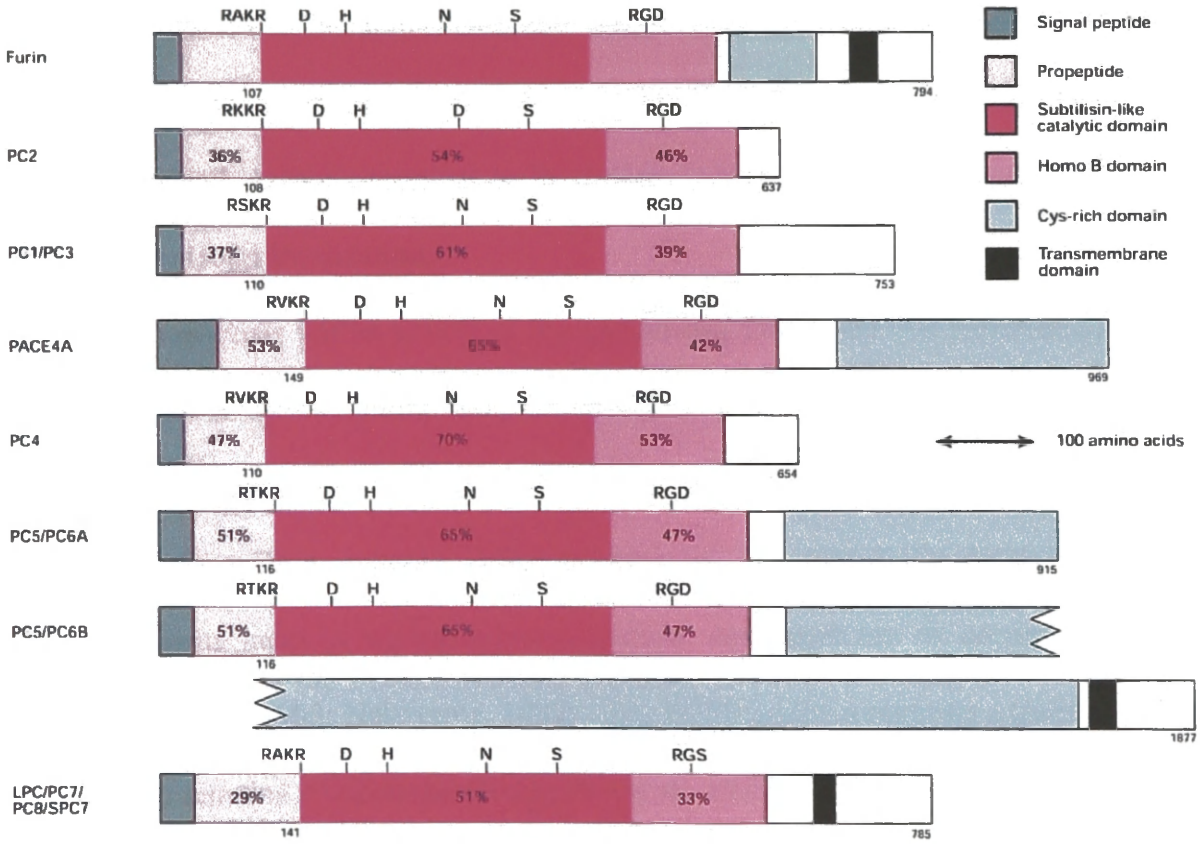


Figure 1B. **Structural comparison of mammalian subtilisin-like pro-protein convertases (as shown in Nakayama 1997).** Schematic representation of the SPCs is shown. The percentage of amino acid identity with furin is shown for each region. Single letter amino acid abbreviations are used to indicate the propeptide cleavage sequence (RXKR), four universally conserved amino acids involved in catalysis (D, H, N, S), and a putative integrin-binding motif (RGD/S).

maturation, thus activating the enzyme. Each of these enzymes is a serine endoprotease with a highly-conserved subtilisin-like catalytic domain containing four universally-conserved residues (aspartate, histidine, asparagine, and serine) directly involved in catalysis. The Homo-B domain (also referred to as the P or middle domain) is also conserved among the SPCs and is essential for enzymatic activity, although its precise function remains unclear. Carboxy-terminal to the Homo-B domain is the cysteine-rich domain (CRD), a region of unknown function found in several of the SPCs, including furin and PACE4. The transmembrane domain (TD), found in several of the SPCs, anchors the enzyme to membranes of the secretory system or to the cell surface. The cytoplasmic tail, carboxy-terminal to the transmembrane domain, contains amino acids that mediate Golgi localization.

Variation exists within structural features among SPCs, and this variation influences subcellular localization. Furin and PC7 contain a transmembrane domain, allowing for localization to the TGN (Nakayama, 1997). Isoforms of PC5, generated through alternative pre-mRNA splicing, differ in structure and subcellular localization. The PC5A isoform lacks a transmembrane domain and localizes to secretory granules (Nakagawa et al., 1993; Nakayama, 1997). The PC5B isoform contains a transmembrane domain and remains intracellular (Nakagawa et al., 1993; Nakayama, 1997). Localization of PACE4 has not been well characterized. Although PACE4 lacks a transmembrane domain, immunochemistry studies suggest that some PACE4 localizes to the TGN (Mains et al., 1997). However, transfection experiments show significant levels of PACE4 being both secreted and retained intracellularly (Mains et al., 1997; Sucic et al., 1999).

Among the widely expressed SPCs, furin and PACE4 are best characterized. Furin processes the widest range of proproteins and recognizes the cleavage sequence motif N-Arg-X-X-Arg-C. All known proproteins containing this motif are processed by furin. PACE4 recognizes a more specific cleavage sequence motif of N-Arg-X-Arg/Lys-Arg-C, and can act upon many, but not all, of the same proproteins as furin. Additionally, not all potential substrates containing this consensus cleavage sequence motif are efficiently processed, suggesting that other factors may be involved with substrate recognition by PACE4. Thus, PACE4 has a more restricted substrate range than does furin. A list of proproteins containing the cleavage sequence motif of PACE4 that are not processed, or are poorly processed, by PACE4 is in Table 1. Amino acids are indicated with their three letter abbreviations.

The three dimensional structure of subtilisin has been solved through X-ray crystallography. This structure was utilized to generate a homology model of the catalytic domain of furin (Van de Ven et al., 1990; Seizen et al., 1994). This molecular modeling, in conjunction with site-directed mutagenesis studies, has led to predicted interactions between substrate amino acids in the P6 through P1' positions and amino acids within the catalytic domain of furin (Seizen et al., 1994). While no molecular model exists for the catalytic domain of PACE4, the 65% amino acid identity between the catalytic domains of PACE4 and furin suggests a similar structure is likely in PACE4. These molecular modeling studies also led to a model of the substrate binding region of furin (Fig. 2). This model shows furin and a bound substrate, with "pockets" of the enzyme occupied by side chains of substrate amino acids. Nomenclature for these pockets is similar to nomenclature for substrate amino acid positions; S (substrate binding pocket) is used

Table 1. Cleavage sequences in proteins not processed, or poorly processed, by PACE4 even though the recognition motif exists

	P4	P3	P2	P1
PACE4	Arg	X	Arg/Lys	Arg

Protein	P6	P5	P4	P3	P2	P1	P1'
Factor IX	Leu	Asn	Arg	Pro	Lys	Arg	Tyr
RPTPμ	Gln	Gln	Arg	Pro	Arg	Arg	Thr
HA	Lys	Lys	Arg	Glu	Lys	Arg	Gly
Avian HA	Arg	Lys	Arg	Lys	Lys	Arg	Gly
NDV F	Gly	Arg	Arg	Gln	Arg	Arg	Phe
gp160	Val	Gln	Arg	Glu	Lys	Arg	Ala
ST3	Arg	Asn	Arg	Gln	Lys	Arg	Phe

The cleavage recognition motif of PACE4, located above the table, and the amino acids around this motif, are defined with specific nomenclature. Substrate residues from the P6 to P1' positions are indicated with their three letter abbreviations. Using pro-factor IX as an example, the P1 (Arg) is carboxy-terminal. Amino-terminal to the P1 is P2 (Lys), amino-terminal to the P2 is P3 (Pro), and amino-terminal to the P3 is P4 (Arg). Carboxy-terminal to the P1, amino acids are designated as P1', P2', P3', etc. Amino-terminal to the P4, amino acids are designated as P5, P6, etc. The site of endoproteolytic cleavage is located between the P1 and P1'. Proteins are as follows: Factor IX (pro-factor IX), RPTPμ (receptor protein tyrosine phosphatase μ), HA (human influenza hemagglutinin), Avian HA (avian influenza hemagglutinin), NDV F (Newcastle disease virus F protein), gp160 (human immunodeficiency virus glycoprotein 160), and ST3 (stromelysin 3). gp160 and ST3 are processed poorly by PACE4; none of the other substrates are processed by PACE4.

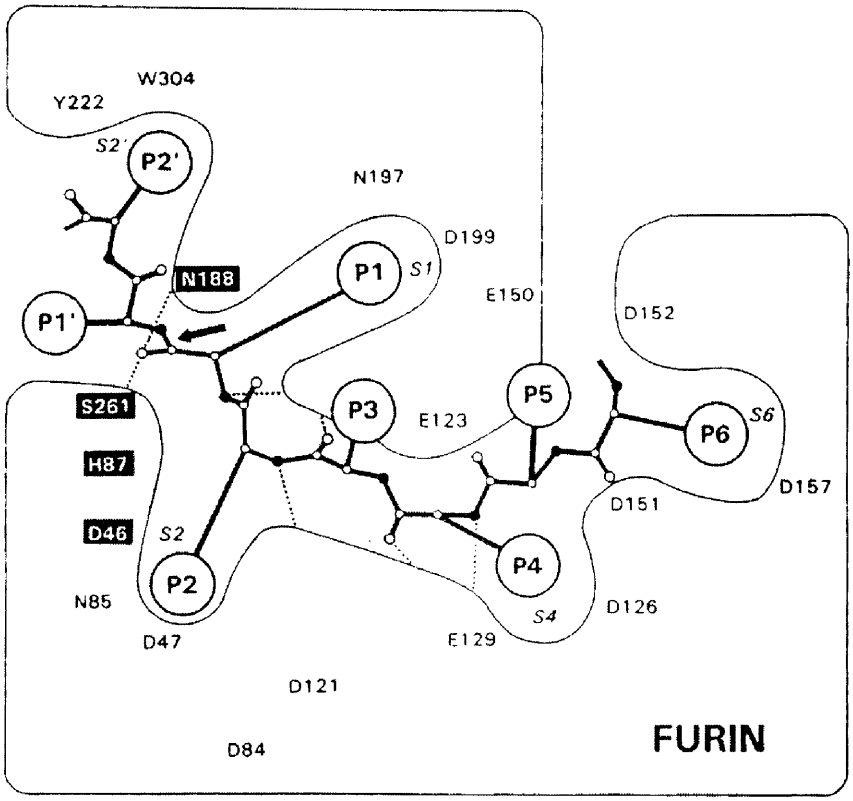


Figure 2. Furin substrate binding region in complex with proprotein model substrate (from Seizen et al. 1994). A schematic two-dimensional model of the substrate binding cleft of furin based on a subtilisin homology model is shown. Thick lines indicate the substrate backbone, thin lines represent furin, and dotted lines represent potentialhydrogen bonds between the backbone and furin. Amino acids of furin are indicated with their single letter abbreviations. Substrate residues from the P6 to P2' positions are shown.

instead of P. The model also indicates residues of furin at or near the pockets that are proposed to interact with side chains of substrate residues in positions P6 through P2'. The four residues highlighted in black boxes are believed to be directly involved in catalysis and are completely conserved among the SPCs. Many other residues are also conserved in all the SPCs, but some variation exists that may affect
the interaction between substrate and enzyme, and in turn play a role in mediating substrate specificity of individual SPCs.

Considering the furin substrate binding region model, and sequences shown in Table 1, some trends for proteins not processed by PACE4 are indicated. Five of the proteins (influenza hemagglutinin, avian influenza hemagglutinin, Newcastle disease virus F protein, human immunodeficiency virus glycoprotein 160, and stromelysin 3) have hydrophobic residues (glycine, phenylalanine, or alanine; indicated in blue) at the P1' position. The model indicates that the side chain of the residue at this position is directed toward the solvent, and does not indicate interactions among residues of furin and the P1'side chain of the substrate residue. However, if a residue with a hydrophobic side chain were at the P1' position, the side chain will be repelled from the solvent. This repulsion may affect overall conformation of the substrate backbone, which could affect interactions between the substrate and enzyme. This modified backbone conformation could impair either the binding of the substrate to the enzyme or the ability of the enzyme to act upon the bound substrate. Either scenario could affect processing by PACE4. Indeed, no known substrates of PACE4 contain a hydrophobic P1' residue. Typically, a polar residue such as serine is found in the P1' position in PACE4 substrates. Three proteins (pro-factor IX, Newcastle disease virus F protein, and human immunodeficiency

virus glycoprotein 160) have residues with hydrophobic side chains (leucine, glycine, or valine; indicated in red) at the P6 position. In the S6 pocket, the model contains two residues having highly charged side chains, aspartic acid (D157; shown in Fig. 2) and lysine 154 (not shown in Fig. 2, but described by Seizen et al.), into close proximity with the side chain of the P6 amino acid. In PACE4, both of these residues are conserved. If the P6 residue side chain is hydrophobic, it would be unable to interact with the highly charged residues of the S6 pocket. In this scenario, the substrate might not bind to the enzyme as efficiently as a substrate with a P6 residue that could interact with residues of the S6 pocket, thus resulting in a decrease in processing efficiency. No known PACE4 substrates contain leucine, glycine, or valine in the P6 position. Finally, the presence of hydrophobic residues in both the P1' and P6 positions, which is the case for two proteins (Newcastle disease virus F protein and human immunodeficiency virus glycoprotein 160) may have an even greater effect on substrate binding or chemical catalytic rate. Two proteins (pro-Factor IX and receptor protein tyrosine phosphatase μ) have a proline at the P3 position (indicated in violet). The unusual side chain of proline prevents free rotation around the amino acid's α carbon and imposes structural rigidity that could impact conformation of the substrate backbone. Such a proline could negatively affect the ability of the substrate to bind to the enzyme or alter the alignment of a bound substrate, thus adversely affecting catalytic activity.

Currently, little is known about the substrate selectivity of PC5 and PC7, although these endoproteases may have restricted substrate ranges compared to furin. From examinations done with protein substrates, PC5 is proposed to have a cleavage sequence motif of N-Arg-X-X-Arg-C (Duguay et al., 1997; Nachtigal and Ingraham, 1996; Cui et

al., 1998). PC7 is also proposed to have an N-Arg-X-X-Arg-C cleavage sequence motif, which was determined through use of synthetic fluorescent substrates (Munzer et al., 1997).

These widely expressed SPCs play critical physiological roles in normal development through activation of several hormones, blood proteins, growth factors, receptors, and proteins of the extracellular matrix (Nakayama, 1997; Seidah and Chretien, 1997; Steiner, 1998). Additionally, these enzymes activate proteins involved in the development of disease conditions, including viral glycoproteins, bacterial toxins, and proteins involved in tumor progression (Nakayama, 1997; Seidah and Chretien, 1997; Steiner, 1998). Specific physiological or pathological roles for individual SPCs have been difficult to determine, as SPCs often show functional redundancy, i.e. a protein can be activated by more than one SPC. However, substrate selectivity is also seen, where only one or two of the SPCs can activate a particular substrate protein. Determination of underlying mechanisms for substrate selectivity of SPCs is of key importance for gaining insight into the role(s) of each SPC. Considering the role of SPCs in activating bacterial toxins and viral proteins, development of specific inhibitors for these enzymes—particularly for antiviral purposes—has also become a focus of intense research interest. Design of specific inhibitors could be greatly facilitated by understanding mechanisms that mediate substrate specificity in SPCs.

I proposed to focus my interests in this study on the hydrophobic residues of the P1' position, to determine if they are involved in substrate selectivity of the four widely expressed SPCs. To test this hypothesis, the precursor of the blood clotting protein, von Willebrand Factor (pro-vWF), a substrate efficiently processed by all four widely

expressed SPCs, was used. Cleavage site residues of pro-vWF, and their numerical positions in the pro-vWF primary structure, are shown in Table 2. Residue substitutions, which incorporated glycine, alanine, and phenylalanine, at the P1' position were generated through use of site-directed mutagenesis.

Expression constructs incorporating these changes, along with expression constructs containing complementary DNA (cDNA) coding for the four widely expressed SPCs, were transfected into RPE.40 cells, a mutant strain of CHO-K1 (Chinese Hamster Ovary Strain K1) cells characterized by a resistance to *Pseudomonas* exotoxin A (PEA) (Moehring and Moehring, 1983). RPE.40 cells harbor mutations within the *fur* alleles that lead to production of non-functional furin; as a result, RPE.40 cells are furin null (Spence et al., 1995; Sucic et al., 1998). These cells are also believed to be PACE4 null (Sucic et al. 1998), but may express minimal levels of PC5 and PC7 (Duguay et al., 1997). Thus, these endoprotease-deficient cells fail to activate PEA and also fail to process many expressed proproteins. RPE.40 cells provide an ideal vehicle for analyzing pro-vWF processing by SPCs, as removal of the propeptide of pro-vWF, essential for function, is not required for secretion. Removal of the propeptide also causes a significant molecular weight shift upon SDS-PAGE, from 350 kilodaltons (kDA) in pro-vWF to 250 kDa in mature vWF. Therefore, conditioned medium containing secreted vWF protein(s) from transfected RPE.40 cells can be analyzed for presence of processing by the four SPCs.

Table 2. Cleavage site sequence and codons of pro-von Willebrand Factor

	P6	**P5**	**P4**	**P3**	**P2**	**P1**	**P1'**
Residue #	758	759	760	761	762	763	764
Residue	Ser	His	Arg	Ser	Lys	Arg	Ser
wt pro-vWF codons	TCT	CAT	CGC	AGC	AAA	AGG	AGC
P1'alanine mutant codons	TCT	CAT	CGC	AGC	AAA	AGG	GCC
P1' phenylalanine mutant codons	TCT	CAT	CGC	AGC	AAA	AGG	TTC
P1'glycine mutant codons	TCT	CAT	CGC	AGC	AAA	AGG	GGC

Residues for pro-vWF from the P6 to P1' positions are indicated with their three letter abbreviations. Codons for each position are shown for wild type (wt) pro-vWF and the three P1' mutants (P1'alanine, P1'phenylalanine, and P1' glycine).

Materials and Methods

Materials

Unless otherwise specified, all enzymes were purchased from Gibco-BRL (Gaithersburg, MD). Unless otherwise specified, all chemicals were obtained at the highest purity available from Fisher Scientific (Pittsburgh, PA).

Plasmids

pBluescipt KS (pBS) was purchased from Stratagene (La Jolla, CA). The cDNA encoding human PACE4 was cloned into the expression plasmid pSVL as described earlier (Sucic et al., 1999). The cDNA encoding wild type CHO-K1 furin was cloned into the expression plasmid pcDNA3 as described previously (Spence et al. 1995). An expression vector directing production of mouse PC5 (the secreted PC5A isoform) was a generous gift from Dr. Kazuhisa Nakayama (Institute of Biological Sciences, University of Tsukuba; Tsukuba, Ibaraki, Japan). An expression vector directing production of mouse PC7 was a generous gift from Dr. Kumar Sambamurti (Department of Pharmacology, Mayo Clinic; Jacksonville, FL). An expression vector directing production of human pro-vWF (pSVL-vWF) was a generous gift from Dr. Jan A. van Mourik and Dr. Jan Voorberg of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

Site-directed Mutagenesis to Incorporate Glycine and Phenyalanine P1' Changes

Site-directed mutagenesis was performed using the QuikChange system (Stratagene). Oligonucleotide mutagenic primers, designed according to QuikChange specifications, were purchased from Gibco-BRL and reconstituted and stored as directed by the supplier. The QuikChange system was used according to manufacturer specifications, with the following recommended modifications from contact with technicians at Stratagene: 170 ng of pSVL-vWF used (as opposed to 50 ng), 250 ng of each oligonucleotide primer used (as opposed to 125 ng), 20 PCR cycles with 1 μL PfuTurbo DNA polymerase added at beginning of 10th PCR cycle (as opposed to 12 PCR cycles and no additional DNA polymerase), and ethanol precipitation and concentration of DNA after mutagenesis reactions and prior to transformation into Epicurian Coli XL1-Blue supercompetent cells (which allowed transformation of all of the DNA from each mutagenesis reaction). These modifications were recommended due to the large size of the pro-vWF cDNA insert [8.8 kilobase pairs (kb)] and the correspondingly large size (13 kb) of the pSVL-vWF construct, as the QuikChange system is optimal for plasmids less than 10 kb.

Glycerol Stocks

Samples of bacterial cells harboring plasmids of interest were grown overnight in Luria-Bertani (LB) broth containing 200 μg/mL ampicillin. Cells were preserved at -70°C after adjusting overnight cultures to 15% glycerol.

Plasmid Preps and Analysis

Colonies derived from transformed Epicurian Coli XL1-Blue cells were propagated in 5 mL LB broth, containing 200 μg/mL ampicillin, at 37°C for ≥ 16 hours. The QIAprep Spin Miniprep Kit (QIAGEN; Valencia, CA) was used to purify plasmid DNA from these cells, following manufacturer instructions. Restriction digests with *Kpn* I were performed and samples were resolved using 1% agarose gels in 0.5x TBE (45 mM **T**ris-**B**orate, 1 mM **E**DTA) to confirm the presence of pSVL-vWF, which shows three discrete bands [7 kb, 4.7 kb, and 1.7 kb] upon *Kpn* I digestion. Automated sequencing to verify presence of the mutations was done by the University of Michigan DNA Sequencing Core Facility (University of Michigan; Ann Arbor, MI). The Concert High Purity Plasmid Midiprep System (Gibco-BRL) was used to purify larger quantities of DNA for subsequent use in transfections. For Midipreps, Epicurian Coli XL1-Blue cells harboring plasmids of interest were propagated in 30 mL LB broth, containing 200 μg/mL ampicillin, at 37° C for ≥ 16 hours with rapid (≥250 rpm) shaking for aeration.

Incorporation of the Alanine P1' Change

Analysis of pro-vWF cDNA for Subcloning and Site-directed Mutagenesis

Incorporation of the alanine P1' change was difficult. Initial attempts were made using the QuikChange system, which was successful for incorporation of the P1' glycine and phenylalanine changes; however, even with the aforementioned modifications, generation of the alanine P1' change was unsuccessful. An alternative strategy was therefore developed that involved subcloning a fragment of pro-vWF cDNA containing the mutagenesis target sequences into pBS (a plasmid of smaller size than pSVL),

incorporating the mutation with site-directed mutagenesis, and reintroducing the mutant fragment back into the pSVL-vWF backbone.

The MapDraw application of Lasergene software (DNASTAR; Madison, Wisconsin) was used to examine the pro-vWF cDNA and pSVL sequences for restriction sites that would allow subcloning of a fragment containing the mutagenesis target sequences. Two *Pvu* I restriction sites were discovered, which produce a 5.5 kb fragment (containing the desired mutagenesis sites) and an 8 kb fragment. These restriction sites are located in the pro-vWF cDNA and the ampicillin resistance (Amp^R) gene carried by pSVL (Fig. 3).

MapDraw (DNASTAR) was also used to examine pBS for *Pvu* I restriction sites that would allow introduction of the pSVL-vWF fragment into pBS. Two restriction sites were found, producing a 1.9 kb backbone fragment and a 1 kb by-product fragment (Fig. 4). Utilizing only *Pvu* I allowed for insertion of the pSVL-vWF fragment into the pBS backbone in two orientations. However, as with pSVL, one of the two *Pvu* I sites in pBS is located in the Amp^R gene, which is an ideal location, as the 5.5 kb fragment from pSVL-vWF can be introduced into pBS in an orientation that will correctly reform the Amp^R gene. Thus, in subsequent transformations, a selection method was in place for "correctly" ligated plasmids, as only transformed cells with the pSVL-vWF fragment in the correct orientation would propagate in the presence of ampicillin.

Restriction Digests and Purification of DNA

To produce the desired fragments for subcloning, restriction digests were performed using *Pvu* I on pSVL-vWF and pBS, and samples were resolved using 1% agarose gels in

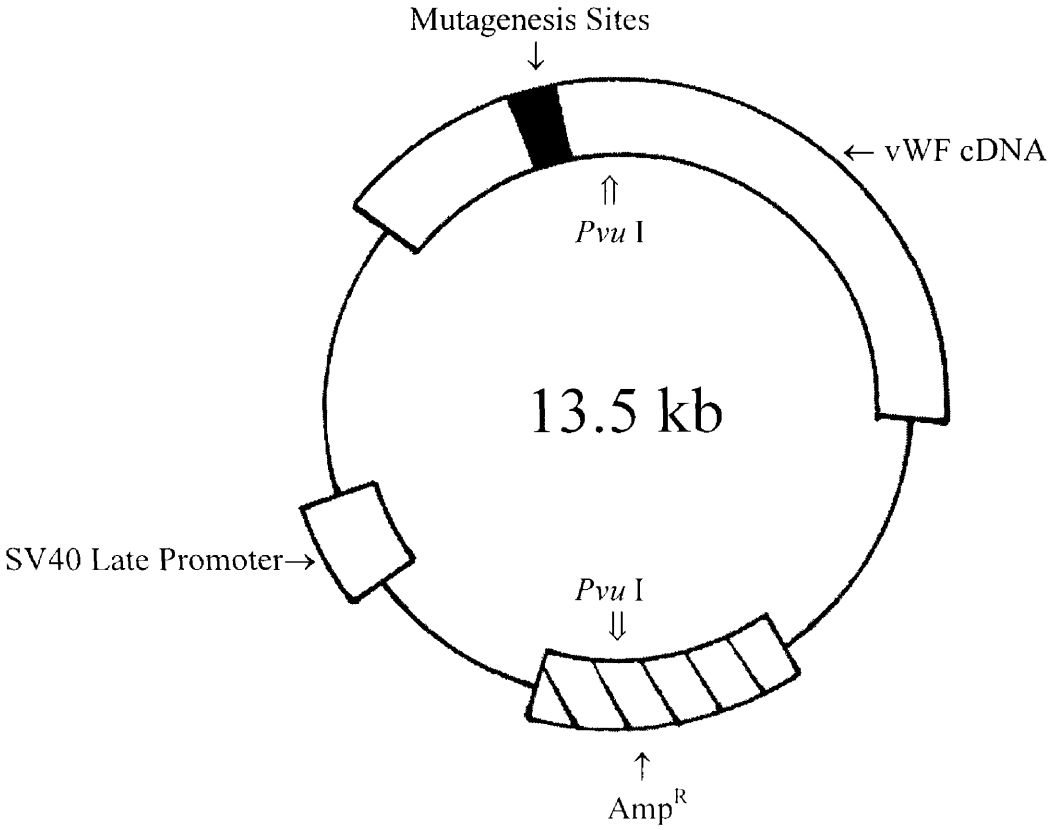


Figure 3. *Pvu* I restriction sites in pSVL-vWF. Two *Pvu* I restriction sites are indicated; one is located in the Amp^R gene, while the other is in the pro-vWF cDNA. Mutagenesis sites for the P1' residue are indicated in black.

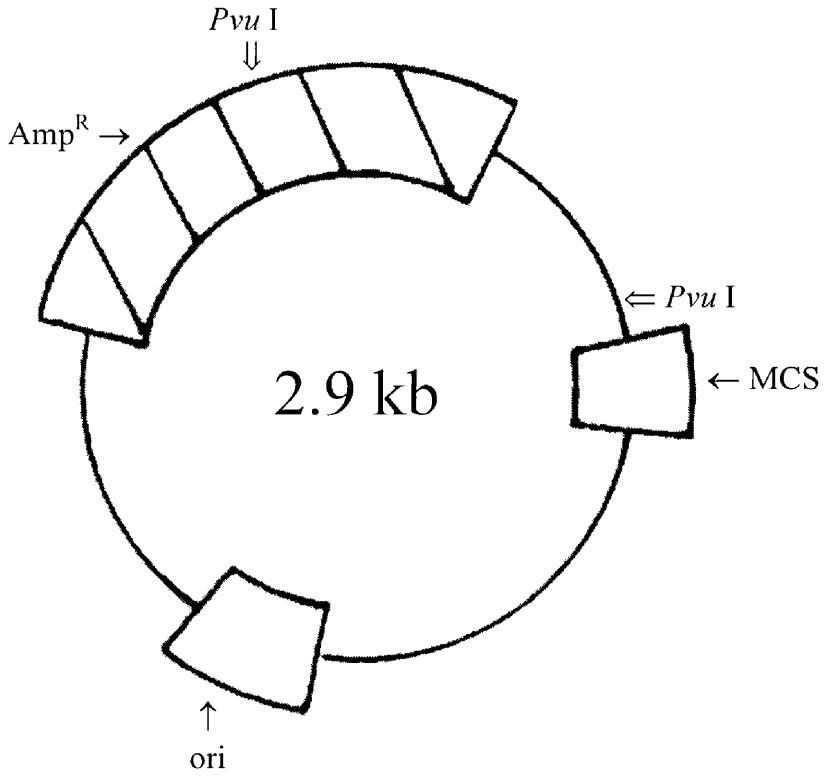


Figure 4. *Pvu* I restriction sites in pBS. Two *Pvu* I restriction sites are indicated; one is located in the Amp^R gene, while the other is near the multiple cloning site (MCS). The origin of replication (ori) is also indicated.

1x TAE (40 mM **T**ris-**A**cetate, 1 mM **E**DTA). Bands representing the fragments of interest were viewed using long wave (320 nm) UV light, excised with a sterile razor blade, and purified utilizing the Wizard PCR Preps DNA Purification System (Promega; Madison, WI) according to manufacturer specifications. Verification of DNA of interest and approximate molar concentrations were determined by examining the purified fragments with a 1% agarose gel (TBE).

Ligations and Transformations

Ligations were performed to incorporate the pSVL-vWF fragment into pBS, using T4 DNA Ligase (Gibco-BRL) and equimolar concentrations of the pBS and pSVL-vWF fragments. Ligation reactions were incubated at 16° C for ≥ 12 hours, and 5 μL of each reaction was used to transform *E. coli* XL1-Blue competent cells produced according to standard protocols (Sambrook et al., 1989). Following addition of ligation products, cells were incubated on ice for 20 minutes, heat shocked at 42° C for 2 minutes, and incubated at 37° C for 1 hour following addition of 950 μL of LB broth. Transformed cells (200 μL) were plated on LB agar, containing 200 μg/mL ampicillin, and grown at 37° C for ≥ 16 hours.

Plasmid Preps and Analysis

Colonies obtained following transformations were used to start cultures (in LB broth containing 200 μg/mL ampicillin) for small scale plasmid isolations. The QIAprep Spin Miniprep Kit (QIAGEN) was used to purify plasmid DNA from ligation reactions. Restriction digests with *Pvu* I were performed and samples were resolved using 1%

agarose gels (TBE) to confirm proper digestion of pBS carrying the pro-vWF insert, which showed two discrete bands (5.5 kb and 1.9 kb) upon *Pvu* I digestion.

Mutagenesis with pBS containing pro-vWF fragment

Site-directed mutagenesis was performed using the QuikChange system (Stratagene). Mutagenesis reactions and subsequent transformations were performed according to manufacturer specifications, with no modifications.

Plasmid Preps and Analysis

Colonies obtained following transformations were used to start cultures (in LB broth, containing 200μg/mL ampicillin) for small scale plasmid isolations. The QIAprep Spin Miniprep Kit (QIAGEN) was used to purify plasmid DNA (pBS with pro-vWF insert) from mutagenesis reactions, as described earlier. Restriction digests with *Pvu* I were performed and samples were resolved using 1% agarose gels (TBE) to confirm proper digestion of pBS with pro-vWF insert. Automated sequencing to verify presence of the mutation was done by the University of Michigan DNA Sequencing Core Facility.

Reintroduction of mutated pro-vWF fragment back into pSVL

Pvu I restriction digests were performed on pSVL-vWF and pBS with pro-vWF insert, and samples were resolved using 1% agarose gels (TAE). Bands on agarose gels containing fragments of interest were viewed using long wave UV light (320 nm), excised with a sterile razor blade, and purified utilizing the Wizard PCR Preps DNA Purification System (Promega). Verification of DNA of interest and approximate molar

concentrations were determined by examining each purified fragment with a 1% agarose gel (TBE).

Ligations and Transformations

Ligations were performed to incorporate the mutant pSVL-vWF fragment back into pSVL, using T4 DNA ligase and equimolar concentrations of DNA, as described earlier. Ligation products were transformed into *E. coli* XL1-Blue cells as described above.

Plasmid Preps and Analysis

Colonies obtained following transformations were used to start cultures (in LB broth, containing 200 μg/mL ampicillin) for small scale plasmid isolations. The QIAprep Spin Miniprep Kit (QIAGEN) was used to purify plasmid DNA from ligation reactions, and restriction digests with *Kpn* I were performed to confirm proper digestion of pSVL-vWF, as described earlier.

Mammalian Cell Culture

CHO-K1 and RPE.40 cells were maintained in Dulbecco's modification to Eagle's Minimal Essential Medium (D-MEM/F-12) supplemented with 7.5% fetal bovine serum (FBS), non-essential amino acids (NEAA; diluted to 1x from a 100x stock obtained from Gibco-BRL), 0.05mg/mL gentamicin, and 0.25 μg/mL fungizone. Cells were maintained in a humidified atmosphere of 4-4.5% CO_2 at 37°C.

Transfections

Either 5 x 10^5 RPE.40 cells were plated in 60 mm x 15 mm (P60) tissue culture dishes or 2 x 10^5 RPE.40 cells were plated in 35 mm x 10 mm (P35) tissue culture dishes. Cells were incubated for 16 hours prior to transfections. Lipofectamine reagent (Gibco-BRL) was used according to manufacturer's instructions, with either 3 μg DNA (P60s) or 1.2 μg DNA (P35s). For coexpression experiments, 3 μg DNA (P60s) of each construct or 1.2 μg DNA (P35s) of each construct was used. Transfected cells were incubated for 60 hours and conditioned medium containing vWF was then collected for analysis.

SDS-PAGE and Western Blotting to Analyze pro-vWF Processing

Sodium **d**odecyl **s**ulfate **p**oly**a**crylamide **g**el **e**lectrophoresis (SDS-PAGE) was performed on conditioned medium samples from transfections, as described previously (Laemmli, 1970), under denaturing conditions with a 5% (w/v) polyacrylamide resolving gel. Resolved proteins were transferred to Immobilon-P membranes (Millipore Corporation; Bedford, MA). Western blot analysis was performed as described previously (Sucic et al., 1998), using a rabbit anti-human vWF polyclonal antibody (DAKO Corporation; Carpinteria, CA), and anti-rabbit IgG alkaline phosphatase conjugate and alkaline phosphatase substrate/detection reagents obtained from Promega.

In vitro Processing Reactions

RPE.40 cells stably over-expressing each of the four SPCs were used as sources of individual enzymes. For reactions requiring PACE4 and PC5, conditioned medium from

RPE.40 cells stably over-expressing these SPCs was used, as cells expressing PACE4 and PC5 secrete large amounts of these endoproteases (Sucic et al., 1999; also unpublished results). Conditioned medium was also used as a source for furin, as a secreted, truncated form of furin was generated and stably over-expressed in RPE.40 cells (Sucic et al., in preparation). Membrane fractions containing fragments of e.r. and Golgi apparatus were used in reactions requiring PC7. Isolation of these fractions was performed on cells stably expressing PC7 (B.A. Ziemba and J.F. Sucic, unpublished) as described previously (Inocencio et al., 1994). Substrates for the reactions were either wild type or mutant forms of pro-vWF generated by RPE.40 cells. Reactions contained 1x *in vitro* processing (IVP) buffer [10 mM HEPES (pH 7.3), 3 mM $CaCl_2$, and 0.1% Triton X-100], substrate proprotein, and equivalent amounts of conditioned medium or membrane fractions containing SPC activity. Reactions were incubated at 37° C for 16 hours using the dwell temperature application of an Amplitron II thermocylcer (Barnstead/Thermolyne; Dubuque, IA). Reaction products were subjected to SDS-PAGE and Western blotting as described above.

Results

In vivo processing of mutant forms of pro-vWF by widely expressed SPCs

Lipofectamine transfection experiments were performed using RPE.40 cells. Mutant forms of pro-vWF, harboring the P1' substitutions, were expressed in RPE.40 cells individually or in tandem with each widely expressed SPC. Transfected cells were incubated for 60 hours, and conditioned medium containing vWF was collected for analysis. Proteins were resolved by SDS-PAGE, and vWF was detected using Western blotting.

Results are shown in Fig. 5. The blot labeled RPE.40 shows the results obtained when cells were transfected only with the various pro-vWF constructs; no SPCs were coexpressed. As a result, no processing of pro-vWF into mature vWF was observed, consistent with earlier work in which pro-vWF had been expressed (Sucic et al., 1998). The remaining four blots show results of coexpression experiments. Interestingly, furin and PACE4 processed wild type pro-vWF and each of the P1' mutants in a consistent pattern. Wild type pro-vWF was completely or nearly completely processed, as was the version of pro-vWF harboring the P1' alanine. Processing of pro-vWF harboring a P1' glycine was slightly impaired, while processing of pro-vWF containing a P1' phenylalanine was more significantly impaired. These results were unexpected, considering that furin has been shown to efficiently process several proteins containing P1' hydrophobic residues, including phenylalanine (Nakayama, 1997). PC5 efficiently processed wild type pro-vWF and the mutant versions containing P1' alanine

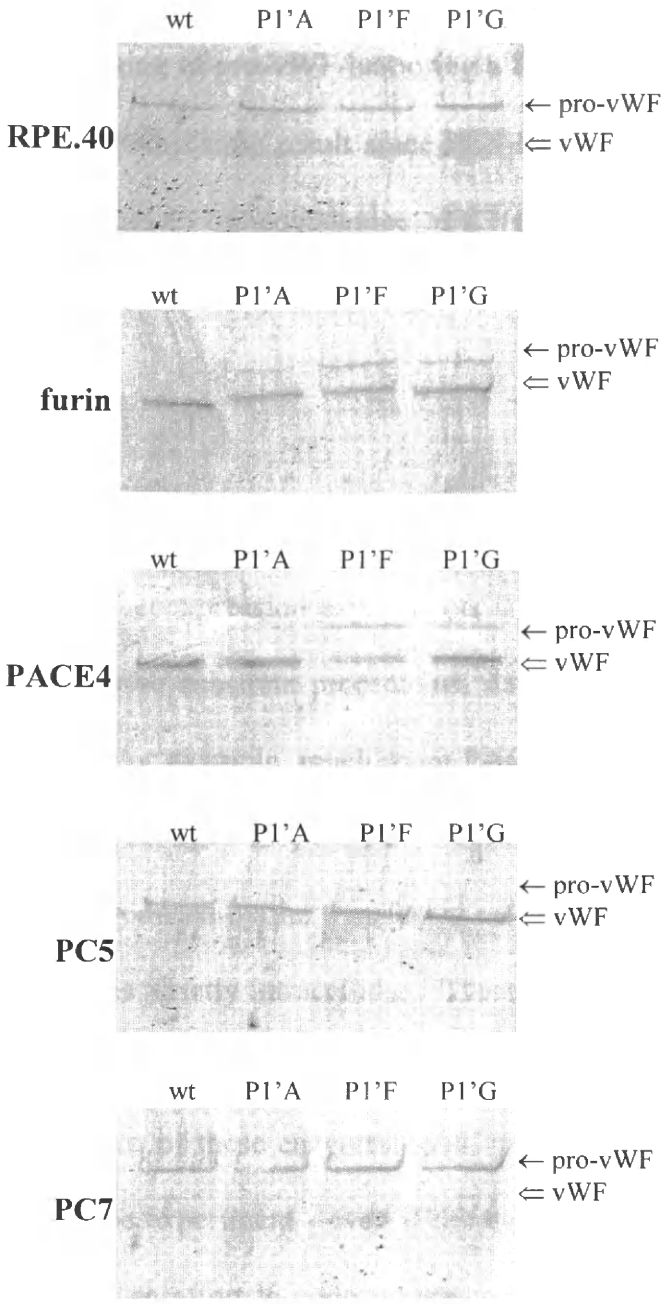


Figure 5. ***In vivo*** **processing of pro-vWF proteins harboring P1' substitutions.** RPE.40 cells were used in transfection experiments in which expression plasmids coding for the various forms of pro-vWF were introduced individually, or in tandem, with expression plasmids coding for one of the SPCs. Cells were incubated for 60 hours, media was collected, proteins were resolved using SDS-PAGE, and vWF was detected using Western blotting. The blot labeled RPE.40 shows results of expressing the forms of pro-vWF in RPE.40 cells without coexpression of an SPC; the remaining four blots state the SPC used. Listed above each blot are the various forms of vWF used. Wild type (wt) vWF is in lane 1; lanes 2-4 contain mutant forms of vWF, in which the P1' residue (S764) has been altered to the residue specified (A-alanine, F-phenylalanine, or G-glycine). Relative positions of pro-vWF and mature vWF are indicated.

and glycine residues. Processing of pro-vWF harboring a P1' phenylalanine was slightly impaired, which was another surprising result since PC5 has been shown to process at least one substrate containing a P1' phenylalanine. PC7 processed all versions of pro-vWF very inefficiently, with no observable differences between the wild type version and the P1' mutants.

In vitro processing of mutant forms of pro-vWF by widely expressed SPCs

An inherent weakness of coexpression experiments is that the different SPCs have varying opportunities to process substrate proproteins, depending upon the subcellular distribution of the enzyme. For example, much more PACE4 is secreted by transfected cells than furin (Sucic et al., 1999). Also, the PC5A isoform was used in coexpression studies, and all of the PC5 produced by the transfected cells was secreted (Nakagawa et al., 1993). In contrast, PC7 is strictly intracellular. Therefore, the processing activities observed for PACE4, PC5, and to some extent furin, may be over-represented relative to PC7, because secreted versions of these enzymes could process secreted pro-vWF over the 60 hour time course of the experiment—even if the version of pro-vWF is a "poor" substrate for the enzyme. In an effort to address this weakness, the ability of SPCs to process the different versions of pro-vWF *in vitro* was examined.

RPE.40 cells stably expressing each of the four SPCs were used as sources of individual enzymes. Conditioned medium was used as sources of PACE4, PC5, and the secreted form of furin. Membrane fractions containing e.r. and Golgi components were used in reactions requiring PC7. These enzyme sources were examined for endoproteolytic activity versus wild type and mutant forms of pro-vWF under previously

described *in vitro* processing reactions (Inocencio et al., 1994). Reaction products were resolved using SDS-PAGE, and vWF was detected using Western blotting.

Results of *in vitro* processing reactions are shown in Fig. 6. The blot labeled RPE.40 is a control, as conditioned medium from untransfected RPE.40 cells was used versus the different version of pro-vWF. As expected, no processing of pro-vWF into mature vWF was observed. The remaining four blots show *in vitro* processing by conditioned medium or membrane fractions from cells stably expressing the SPCs. Very similar *in vivo* processing was observed for furin, PACE4, and PC5. Each enzyme showed significant processing of wild type pro-vWF. Processing of pro-vWF harboring a P1' alanine or P1'glycine appeared to be slightly impaired, while processing of pro-vWF containing P1' phenylalanine was significantly impaired. Results of *in vitro* processing reactions using PC7 showed approximately equivalent levels of processing of all four substrates, as was observed in results from *in vivo* experiments. These results may indicate a broader substrate range for PC7, with the P1' pro-vWF mutants having no negative effect on processing.

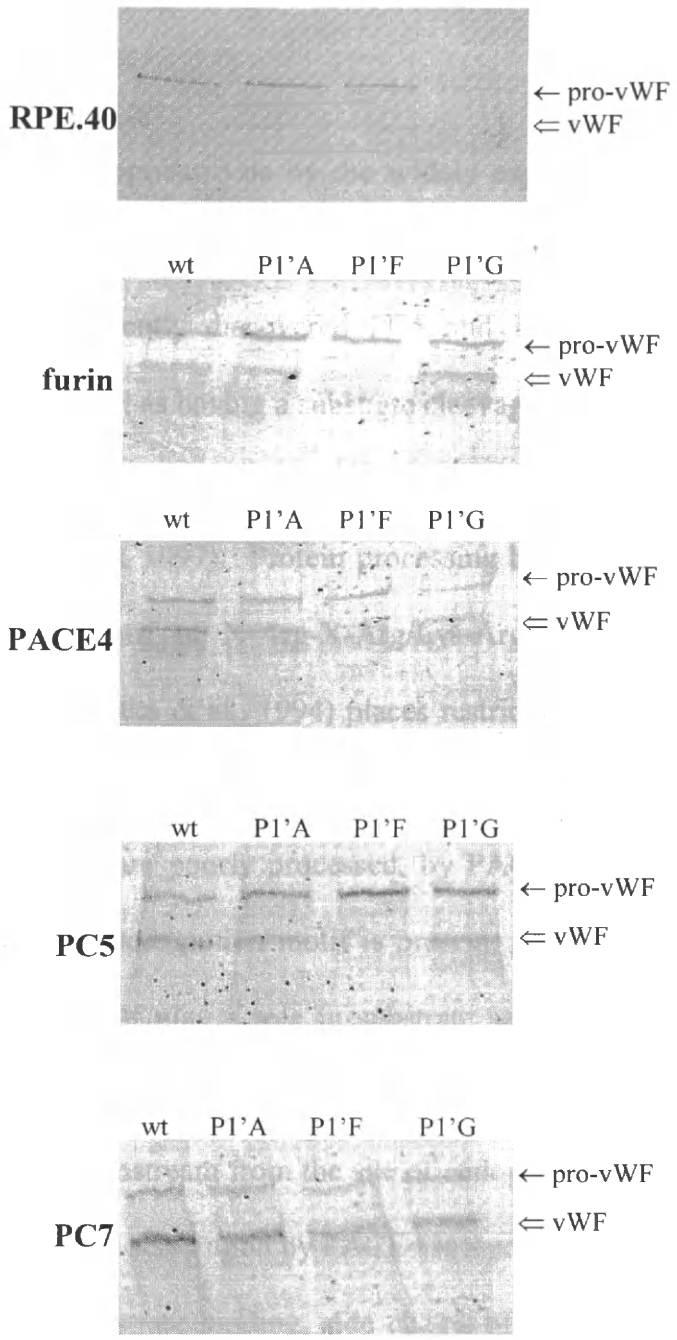


Figure 6. ***In vitro*** **processing of pro-vWF proteins harboring P1' substitutions.** RPE.40 cells stably expressing each of the four SPCs were used as sources of individual enzymes and the various forms of pro-vWF. Conditioned medium was used as sources of PACE4, PC5, and secreted furin. Membrane fractions were used in reactions requiring PC7. Reactions were incubated for 16 hours, proteins were resolved using SDS-PAGE, and vWF was detected using Western blotting. Listed above each blot are the various forms of vWF used. Wild type (wt) vWF is in lane 1. Lanes 2-4 contain mutant forms of vWF, in which the P1' residue (S764) has been altered to the residue specified (A-alanine, F-phenylalanine, or G-glycine). The blot labeled RPE.40 shows control results in which conditioned medium from RPE.40 cells was used; the rest of the blots state the SPC used. Relative positions of pro-vWF and mature vWF are indicated.

Discussion

Recent research on endoproteolysis by the widely expressed eukaryotic SPCs has mostly focused on two members, furin and PACE4, while much less characterization has been done on the more-recently discovered PC5 and PC7. Furin has been well-characterized, and is described as having a substrate cleavage recognition motif of N-Arg-X-X-Arg-C (Nakayama, 1997); when this motif is present, furin efficiently processes the substrate proteins (Nakayama, 1997). Protein processing by PACE4 is more limited, as its cleavage recognition motif of N-Arg-X-Arg/Lys-Arg-C (Creemers et al., 1993; Rehemtulla et al., 1993; Hosaka et al., 1994) places restrictions on substrate specificity. Furthermore, several potential substrates that contain the PACE4 cleavage recognition motif are not processed, or are poorly processed, by PACE4 (Table 1). This lack of processing, even though the recognition motif is present, suggests that residues outside the cleavage recognition motif play a role in substrate selectivity. Five of these non-processed substrates contain a hydrophobic residue (alanine, phenylalanine, or glycine) in the P1' position, directly downstream from the site of endoproteolytic cleavage, while the P1' residue in most substrates acted upon by PACE4 is a polar residue like serine. I have hypothesized that during substrate binding, side chains of hydrophobic residues in this position are repelled from the solvent, thus affecting the overall conformation of the substrate backbone and affecting interactions between the substrate and the enzyme. Changing the nature of these interactions could result in either inefficient substrate processing, or complete abolition of processing, by the enzyme. To determine the role of the amino acid directly downstream from the site of endoproteolytic cleavage, P1' amino

acid substitutions were made in pro-vWF, a substrate processed by all four of the widely expressed SPCs. Three mutant forms of pro-vWF, harboring P1' alanine, P1' phenylalanine, or P1' glycine residues, were generated via site directed mutagenesis (Table 2, page 12). These mutant forms of pro-vWF were examined as substrates for furin, PACE4, and the two other widely expressed SPCs, PC5 and PC7. It has been proposed that PC5 and PC7 have a cleavage sequence motif of N-Arg-X-X-Arg-C (Duguay et al., 1997; Nachtigal and Ingraham, 1996; Cui et al., 1998; Munzer et al., 1997), although little information is currently available about the substrate range of these enzymes.

Results from experiments *in vivo* and *in vitro* suggest that a P1' hydrophobic residue impacts processing. Surprisingly, processing of mutant forms of pro-vWF by furin and PACE4 was nearly indistinguishable (Fig. 5 and 6). These SPCs completely, or nearly completely, cleaved the P1' alanine and P1' glycine pro-vWF mutants, but processing of the P1' phenylalanine mutant was significantly impaired. Nonetheless, the presence of a phenylalanine in the P1' position, by itself, does not appear to play a role in substrate selectivity, at least with the pro-vWF substrate. While Newcastle disease virus F protein, which also contains a P1' phenylalanine, is not processed by PACE4 (Fujii et al., 1999), and stromelysin 3, which likewise contains a P1' phenylalanine, is poorly processed by PACE4 (Santavicca et al., 1996), both substrates are cleaved by furin. These findings, in conjunction with my results, suggest that substrate residues or broader structural features other than the P1' residue and the cleavage recognition motif are playing a role in substrate selectivity. Further experimentation is needed to reconcile the results obtained with the pro-vWF proteins carrying P1' substitutions and results generated in other

laboratories with naturally-occurring substrates. Further experimentation is also needed to determine the precise biochemical basis of the impaired processing observed with the P1' phenylalanine substitution. The presence of the phenylalanine may impact substrate binding or may decrease catalytic efficiency of the SPC on the bound substrate.

Processing of wild type and mutant forms of pro-vWF by PC5 also yielded an unexpected result, observed both *in vivo* and *in vitro*. PC5 efficiently processed wild type pro-vWF and the mutant versions harboring a P1' alanine or glycine, but processing of pro-vWF containing a P1' phenylalanine was impaired. In contrast to these findings, published results show that PC5 can process Newcastle disease virus F protein, which contains a P1' phenylalanine (Fujii et al., 1999). My results, and these published results for substrate processing by PC5, suggest, as was earlier stated, that structural features other than the P1' residue and the cleavage recognition motif are playing a role in substrate specificity.

The processing of the pro-vWF mutants harboring the P1' alanine, P1' phenylalanine, and P1' glycine by furin, PACE4, and PC5, suggest possible courses for future experimentation. Analysis of the P6 substrate residues of Newcastle disease virus F protein and human immunodeficiency virus glycoprotein 160 indicates the presence of a hydrophobic residue (glycine and valine, respectively), while both of these substrates also have a P1' hydrophobic residue (phenylalanine and alanine, respectively). This combination of hydrophobic P1' and P6 residues may play a more important role in mediating substrate selectivity in these SPCs, in contrast to the presence of a P1' hydrophobic residue alone. Using pro-vWF as a substrate, substitutions of the P6 residue with hydrophobic residues, in combination with the three hydrophobic mutants generated

in this study, could be tested against each of widely expressed SPCs. Indeed, this experimentation is a long-term goal of the research in Dr. Sucic's laboratory.

The substrate range and specificity of PC7 have not yet been extensively investigated. Results from this study suggest PC7 may have a broader substrate range than the other widely expressed SPCs. Although not well demonstrated *in vivo*, *in vitro* processing of the three P1' mutant forms of pro-vWF by PC7 was equivalent to that of wild type pro-vWF, in contrast to the results obtained with furin, PACE4, and PC5 (Fig. 5 and 6). The apparent lack of processing of the various forms of pro-vWF by PC7 *in vivo* was likely due to the limited interaction of pro-vWF and PC7 in the Golgi apparatus. Recall that unlike the other SPCs used in this study, PC7 is exclusively localized intracellularly and therefore may have less opportunity to act upon pro-vWF in coexpression experiments.

RPE.40 cells are endoprotease deficient, but can process some proproteins. PC7 is a likely candidate for carrying out these activities, as RPE.40 cells express low levels of PC7 (Duguay et al., 1997). RPE.40 cells have been shown to endoproteolytically cleave the β-site amyloid precursor protein cleaving enzyme, also called the Alzheimer's β secretase (Pinnix et al., 2001), and pro-insulin-like growth factor 1 (Duguay et al. 1997). Diphtheria toxin, known to be processed by furin and PACE4 (Nakayama, 1997; Sucic et al., 1999), may also be processed by PC7, as RPE.40 cells are sensitive to diphtheria toxin (Moehring and Moehring, 1983). Direct processing of these substrates by PC7 has not been demonstrated, and will need to be investigated in future studies. Alternatively, representation of these substrates, by incorporation of amino acid substitutions into pro-

vWF, may allow the substrate specificity of PC7 to be further characterized, through *in vivo* and *in vitro* techniques like those utilized in these studies.

Another substrate processed by RPE.40 cells is the human immunodeficiency virus glycoprotein 160, which has been thoroughly studied. Upon cleavage and subsequent activation by cellular endoproteases into gp120 and gp41, the envelope glycoprotein initiates infection through fusion of the viral envelope with the cell membrane (Hallenberger et al., 1992). Early *in vivo* studies had implicated furin as important for the cleavage of gp160 (Hallenberger et al., 1992; Decroly et al., 1994; Gu et al., 1995). More recent work suggested that furin is not the primary enzyme found to be processing gp160. Processing of gp160 was not increased when furin was overexpressed in RPE.40 cells, and *in vitro* evidence for a calcium-independent gp160 processing enzyme was obtained from *in vitro* reactions (Inocencio et al., 1997). Examination of lymphatic tissues and cells, which interact with gp160 in the development of HIV infection, showed the presence of furin and PC7, but no other SPCs. Coexpression experiments, utilizing these natural host cells, with gp160 and furin or PC7, demonstrated gp160 processing by both SPCs (Hallenberger et al. 1997). The likely presence of PC7 in RPE.40 cells could then explain their processing of gp160.

Future experimentation would allow examination of the effects of a P3 proline on substrate selectivity. Two substrates not processed by PACE4, pro-factor IX and receptor protein tyrosine phosphatase μ, have a P3 position proline. These proteins are processed by furin, while processing of these proteins by PC5 and PC7 is unknown. Incorporation of a single residue substitution in the P3 position of pro-vWF, and a combination of a P3

position proline with a hydrophobic residue in the P6 position, as is found in the substrate pro-factor IX, would allow determination of processing by PACE4, PC5, and PC7.

This study has initiated analysis of substrate selectivity of the SPCs, and the effects of residues outside the cleavage recognition motif. Results demonstrate that the presence of a P1' hydrophobic residue alone is not involved in substrate specificity of some of the widely expressed SPCs. Activity of furin, PACE4, and PC5 was significantly impaired by the presence of a P1' phenylalanine. These results, in conjunction with studies from other laboratories, suggest that the residue residing in the P1'position does not solely determine substrate specificity of the SPCs. These results suggest that further investigation is required to determine the effects of combinations of hydrophobic residues, in the P1' and P6 residue locations, on substrate selectivity of the SPCs. In addition, effects of proline in the P3 position, and studies focused upon further characterization of PC7, can now be undertaken.

Literature Cited

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson. 1994. Molecular Biology of the Cell. Garland Publishing, Inc., New York, NY.

Barr, P.J. 1991. Mammalian subtilisins: the long-sought dibasic processing endoproteases. *Cell*, 66:1-3.

Bassi, D.E., H. Mahloogi, and A.J.P. Klein-Szanto. 2000. The proprotein convertases furin and PACE4 play a significant role in tumor progression. *Mol. Carcinog.*, 28:63-69.

Bresnahan, P.A, R. Leduc, L. Thomas, J. Thorner, H.L. Gibson, A.J. Brake, P.J. Barr, and G. Thomas. 1990. Human *fur* gene encodes a yeast *KEX2*-like endoprotease that cleaves pro-β-NGF *in vivo*. *J. Cell Biol.*, 111:2851-2859.

Creemers, J.W.M., P.J.G. Kormelink, A.J.M. Roebroek, K. Nakayama, W.J.M. Van de Ven. 1993. Proprotein processing activity and cleavage site selectivity of the Kex2-like endoprotease PACE4. *FEBS Lett.*, 336:65-69.

Cui, Y., F. Jean, G. Thomas, and J.L. Christian. 1998. BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. *EMBO J.*, 17:4735-4743.

Decroly, E., M. Vanderbranden, J.M. Ruysschaert, J. Cogniaux, G.S. Jacob, S.C. Howard, G. Marshall, A. Kompelli, A. Basak, F. Jean, and N.G. Seidah. 1994. The convertases furin and PC1 can cleave both the immunodeficiency virus HIV-1 envelope glycoprotein gp160 into gp120 (HIV-1 SU) and gp41 (HIV-1 TM). *J. Biol. Chem.*, 269:12240-12247.

Duguay, S.J., W.M. Milewski, B.D. Young, K. Nakayama, and D.F. Steiner. 1997. Processing of wild-type and mutant proinsulin-like growth factor-1A by subtilisin-related proprotein convertases. *J. Biol. Chem.*, 272:6663-6670.

Fujii, Y., T. Sakaguchi, K. Kiyotani, and T. Yoshida. 1999. Comparison of substrate specificities against the fusion glycoprotein of virulent Newcastle Disease Virus between a chick embryo fibroblast processing protease and mammalian subtilisin-like proteases. *Microbiol. Immunol.*, 43:133-140.

Fuller, R. S., R.E. Sterne, and J. Thorner. 1988. Enzymes required for yeast prohormone processing. *Annu. Rev. Physiol.*, 50:345-362.

Fuller, R.S., A.J. Brake, and J. Thorner. 1989. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science*, 246:482-486.

Gu, M., J. Rappaport, and S.H. Leppla. 1995. Furin is important but not essential for the proteolytic maturation of gp160 of HIV-1. *FEBS Lett.*, 365:95-97.

Hallenberger, S., V. Bosch, H. Angliker, E. Shaw, H.-D. Klenk, and W. Garten. 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein 160. *Nature*, 360:358-361.

Hallenberger, S., M. Moulard, M. Sordel, H.-D. Klenk, and W. Garten. 1997. The role of eukaryotic subtilisin-like endoproteases for the activation of human immunodeficiency virus glycoproteins in natural host cells. *J. Virol.*, 71:1036-1045.

Hosaka, M., K. Murakami, and K. Nakayama. 1994. PACE4A is a ubiquitous endoprotease that has similar but not identical substrate specificity to other Kex2-like processing endoproteases. *Biomed. Res.*, 15:383-390.

Inocencio, N.M., J.F. Sucic, J.M. Moehring, M.J. Spence, and T.J. Moehring. 1997. Endoprotease activities other than furin and PACE4 with a role in processing of HIV-1 gp160 glycoproteins in CHO-K1 cells. *J. Biol. Chem.*, 272:1344-1348.

Inocencio, N. M., J.M. Moehring, and T.J. Moehring. 1994. Furin activates *Pseudomonas* exotoxin A by specific cleavage *in vivo* and *in vitro*. *J. Biol. Chem.*, 269:31831-31835.

Kalafatis, M., J.O. Eagen, C. van 't Veer, K.M. Cawthern, and K.G. Mann. 1997. The regulation of clotting factors. *Crit. Rev. Eukaryot. Gene Expr.*, 7:241-280.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227:680-685.

Lewin, B. 2000. Genes VII. Oxford University Press, New York, NY.

Mains, R.E., C.A. Berard, J.-B. Denault, A. Zhou, R.C. Johnson, and R. Leduc. 1997. PACE4: a subtilisin-like endoprotease with unique properties. *Biochem. J.*, 321:587-593.

Mizuno, K., T. Nakamura, T. Ohsima, S. Tanaka, and H. Matsuo. 1988. Yeast *KEX2* gene encodes an endopeptidase homologous to subtilisin-like serine proteases. *Biochem. Biophys. Res. Commun.*, 156:246-254.

Moehring, J.M., and T.J. Moehring. 1983. Strains of CHO-K1 cells resistant to *Pseudomonas* exotoxin A and cross-resistant to diphtheria toxin and viruses. *Infect. Immun.*, 41:998-1009.

Munzer, J.S., A. Basak, M. Zhong, A. Mamarbachi, J. Hamelin, D. Savaria, C. Lazure, S. Benjannet, M. Chretien, and N.G. Seidah. 1997. *In vitro* characterization of the novel proprotein convertase PC7. *J. Biol. Chem.*, 272:19672-19681.

Nachtigal, M.W. and H.A. Ingraham. 1996. Bioactivation of Mullerian inhibiting substance during gonadal development by a kex2/subtilisin-like endoprotease. *Proc. Natl. Acad. Sci. U.S.A.*, 93:7711-7716.

Nakagawa, T., K. Murakami, and K. Nakayama. 1993. Identification of an isoform with an extremely large cys-rich region of PC6, Kex2-like processing endoprotease. *FEBS Lett.*, 327:165-171.

Nakayama, K. 1997. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem. J.*, 327:625-635.

Pinnix, I., J.E. Council, B. Roseberry, L. Onstead, W. Mallender, J. Sucic, and K. Sambamurti. 2001. Convertases other than furin cleave beta-secretase to its mature form. *FASEB J.*, 15:1810-1812.

Rehemtulla, A., P.J. Barr, C.J. Rhodes, and R.J. Kaufman. 1993. PACE4 is a member of the mammalian prepeptidase family that has overlapping but not identical substrate specificity to PACE. *Biochemistry*, 32:11586-11590.

Roebroek, A.J., J.A. Schalken, M.J. Bussemakers, H. van Heerikhuizen, C. Onnekink, F.M. Debruyne, H.P. Bloemers, and W.J. Van de Ven. 1986a. Characterization of human c-fes/fps reveals a new transcription unit (fur) in the immediately upstream region of the proto-oncogene. *Mol. Biol. Rep.*, 11:117-125.

Roebroek, A.J., J.A. Schalken, J.A. Leunissen, C. Onnekin, H.P. Bloemers, and W.J. Van de Ven. 1986b. Evolutionarily conserved close linkage of the c-fes/fps proto-oncogene and genetic sequences encoding a receptor-like protein. *EMBO. J.*, 5:2197-2202.

Sambrook, J., E.F. Fritsch, and T. Maniatas. 1989. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, NY.

Santavicca, M., A. Noel, H. Angliker, I. Stoll, J.-P. Segain, P. Anglard, M. Chretien, N. Seidah, and P. Basset. 1996. Characterization of structural determinants and molecular mechanisms involved in pro-stromelysin-3 activation by 4-aminophenylmercuric acetate and furin-type convertases. *Biochem. J.*, 315:953-958.

Seidah, N.G., and M. Chretien. 1997. Eukaryotic protein processing: endoproteolysis of precursor proteins. *Curr. Opin. Biotechnol.*, 8:602-607.

Siezen, R.J., J.W.M. Creemers, and W.J.M. Van de Ven. 1994. Homology modelling of the catalytic domain of mammalian furin. *Eur. J. Biochem.*, 222:255-266.

Spence, M.J., J.F. Sucic, B.T. Foley, and T.J. Moehring. 1995. Analysis of mutations in alleles of the *fur* gene from an endoprotease-deficient Chinese hamster ovary cell strain. *Somat. Cell. Molec. Genet.*, 21:1-18.

Steiner, D.F., S.P Smeekens, S. Ohagi, and S.J. Chan. 1992. The new enzymology of precursor processing endoproteases. *J. Biol. Chem.*, 267:23435-23438.

Steiner, D.F. 1998. The proprotein convertases. *Curr. Opin. Chem. Biol.*, 2:31-39.

Sucic, J.F., J.M. Moehring, N.M. Inocencio, J.W. Luchini, and T.J. Moehring. 1999. Endoprotease PACE4 is Ca^{2+}-dependent and temperature sensitive and can partly rescue the phenotype of a furin-deficient cell strain. *Biochem. J.*, 339:639-647.

Sucic, J.F., M.J. Spence, and T.J. Moehring. 1998. Structural and functional analysis of protein products derived from mutant *fur* alleles in an endoprotease-deficient Chinese hamster ovary cell strain. *Somat. Cell. Molec. Genet.*, 24:75-90.

Sucic, J.F., L. Shomali, K. Hockman, D. Beebe, G.M. Gehrke, and H.R. Sucic. Cysteine residues in the catalytic domain of furin form disulfide bonds that are necessary for enyymatic activity. In preparation.

Wise, R.J., P.J. Barr, P.A. Wong, M.C. Kiefer, A.J. Brake, and R.J. Kaufman. 1990. Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proc. Natl. Acad. Sci.*, 87:9378-9382.

Van de Ven, W.J.M., J. Voorberg, R. Fontijn, H. Pannekoek, A.M.W. van den Ouweland, H.L.P. van Duijnhoven, A.J.M. Roebroek, and R.J. Siezen. 1990. Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. *Mol. Biol. Rep.*, 14:265-275.